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Indian Standard

METHODS FOR ESTIMATION OF RIBOFLAVIN (VITAMIN B_2) IN FOODSTUFFS

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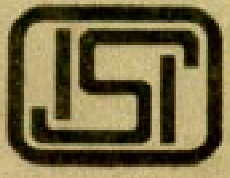

INDIAN STANDARDS INSTITUTION
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 1

May 1970

Indian Standard

METHODS FOR ESTIMATION OF RIBOFLAVIN (VITAMIN B_2) IN FOODSTUFFS

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Indian Standard

METHODS FOR ESTIMATION OF RIBOFLAVIN (VITAMIN B_2) IN FOODSTUFFS

0. FOREWORD

0.1 This Indian Standard was adopted by the Indian Standards Institution on 18 November 1969, after the draft finalized by the Food Hygiene, Sampling and Analysis Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Vitamins are required to be assessed in a large number of foodstuffs, such as dairy products, animal feeds, processed cereals and other foodstuffs. Moreover, different methods of vitamin assay are used in different laboratories. Therefore, with a view to establishing uniform procedures and also for facilitating a comparative study of results, ISI is bringing out a series of standards on vitamin assays. These would include chemical and microbiological methods, wherever applicable.

0.3 This standard covers two methods commonly used for the estimation of riboflavin in foodstuffs. Two methods have been specified, namely, chemical method and microbiological method. Depending upon the available facilities and applicability, either of the methods may be used. It is desirable that the method used is stated in the test report.

0.4 In the preparation of this standard, considerable assistance has been derived from a number of standard books and publications. However, the methods included in this standard are predominantly those which have been tried in various laboratories in the country. Thus the methods prescribed in this standard are mainly based on practical experience within the country.

0.5 In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS: 2-1960*.

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1. SCOPE

1.1 This standard specifies chemical as well as microbiological methods for the estimation of riboflavin (Vitamin B_2) in foodstuffs.

*Rules for rounding off numerical values (*revised*).

2. QUALITY OF REAGENTS

2.1 Unless specified otherwise, pure chemicals shall be employed in tests and distilled water (*see* IS: 1070-1960*) shall be used when the use of water as a reagent is intended.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the result of analysis.

3. PREPARATION OF ASSAY SAMPLE

3.0 The technique used for preparing the material for the analysis is mostly common to every vitamin determination. It should be ensured that the sample taken for the assay is representative of the whole, and any deterioration of the vitamin to be examined is prevented.

3.1 Powders and liquids should be mixed thoroughly until homogeneity is achieved. Dry materials, such as bread, biscuits and grains, should be ground to produce a fine powder.

3.2 Butter should be melted under constant stirring. Samples from margarine or cheese or other such foods should contain portions of the surface as well as of the interior.

3.3 Wet or fresh material may be minced with a knife or scissors, or homogenized in a blender, if necessary, in the presence of the extracting solvent.

4. CHEMICAL METHOD

4.0 Principle — Riboflavin fluoresces when exposed to light of wavelength 440 to 500 mμ. The intensity of the fluorescence is proportional to the concentration of riboflavin in dilute solutions. Riboflavin phosphate (FMN) and flavin adenine dinucleotide exhibit the same characteristic yellow colour and yellow green fluorescence as riboflavin. The riboflavin is measured in terms of difference between the fluorescence before and after chemical reduction by hydrosulphite which will reduce riboflavin and its co-enzymes to colourless compounds which do not fluoresce.

4.1 Apparatus

4.1.1 *Photo-Fluorometer* — Use a fluorometer suitable for accurately measuring fluorescence of solutions containing riboflavin in concentrations of 0·05 to 0·20 μg/ml. A fluorometer having the input filter of narrow transmittance with maximum of about 440 mμ and the output filter of

*Specification for water, distilled quality (*revised*).

narrow transmittance with a maximum of about 565 mμ has been found satisfactory.

4.2 Reagents

4.2.1 *Standard Hydrochloric Acid* — 0·1 N.

4.2.2 *Sodium Hydroxide Solution* — 4 percent (*w/v*).

4.2.3 *Dilute Hydrochloric Acid* — 1:1 (*v/v*).

4.2.4 *Riboflavin Stock Solution* I — Add 50 mg of USP riboflavin reference standard or equivalent IP standard previously dried and stored in dark in a desiccator over phosphorus pentoxide to about 300 ml of 0·02 N acetic acid and warm the mixture on a steam-bath with constant stirring until the riboflavin is completely dissolved, cool and then add 0·02 N acetic acid to make the volume to 500 ml. Store the solution under toluene in the cold in a dark bottle. One millilitre of this solution is equivalent to 100 μg of riboflavin.

4.2.5 *Riboflavin Stock Solution* II — To 50 ml of the riboflavin stock solution I, add 0·02 N acetic acid solution to make 500 ml. Store the solution under toluene in the cold in a dark bottle. One millilitre of this solution is equivalent to 10 μg of riboflavin.

4.2.6 *Standard Riboflavin Solution* — Dilute 10 ml of the riboflavin stock solution II with water to make 100 ml. One millilitre of this solution is equivalent to one microgram of riboflavin. Prepare this solution freshly for each assay.

4.2.7 *Acetic Acid* — Glacial.

4.2.8 *Potassium Permanganate Solution* — Dissolve 4 g of potassium permanganate crystals in 100 ml of water, keep for a few days, filter and store in a dark bottle.

4.2.9 *Hydrogen Peroxide Solution* — 3 percent.

4.2.10 *Sodium Hydrosulphite* — of high purity, unexposed to light or air.

4.3 Procedure

4.3.0 *Caution* — Throughout the procedure, keep the *p*H of the solution below 7·0 to prevent loss of riboflavin.

4.3.1 *Preparation of Sample Solution* — Take a weighed quantity of the sample in a flask of suitable size and add to it a quantity of the standard hydrochloric acid equal in millilitres to not less than 10 times the dry weight of the sample in grams so that the resulting solution shall not contain more than 0·1 mg of riboflavin per millilitre. If the material is not readily soluble, then comminute the sample so that it may be evenly dispersed in liquid. Then agitate the solution vigorously

and wash down the sides of the flask with the standard hydrochloric acid. Heat the mixture in an autoclave at 121° to 123°C for 30 minutes and cool to room temperature. If lumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust with vigorous agitation the *p*H of the mixture to 6·0 to 6·5 with the sodium hydroxide solution. Then immediately add dilute hydrochloric acid until no further precipitation occurs. Dilute the mixture to a known volume so that one millilitre of the final mixture contains more than 0·1 μg of riboflavin. Filter the mixture through a filter paper (Whatman No. 1 or equivalent) which does not absorb riboflavin. In case the mixture is difficult to filter, centrifuge it and filter through fritted glass, using suitable analytical filter-aid which may often be substituted for, or preceded by filtering through a filter paper. Take an aliquot of the clear filtrate and check for dissolved protein by adding dropwise, first dilute hydrochloric acid and if no precipitate forms, then the sodium hydroxide solution with vigorous agitation and proceed as follows:

a) If no further precipitation occurs, adjust the *p*H of the solution to 6·8 by adding the sodium hydroxide solution accompanied by vigorous agitation. Dilute the solution to a known volume so that one millilitre of the solution contains about 0·10 μg of riboflavin. If cloudiness appears, filter again.

b) If further precipitation occurs, adjust the solution again to the point at which the maximum precipitation occurs. Dilute the solution to a known volume so that one millilitre of the solution contains more than 0·10 μg of riboflavin. Filter and take the aliquot of the clear filtrate and proceed further as described above.

4.3.2 *Estimation*

4.3.2.1 Take two tubes (or reaction vessels) and add 10 ml of the sample solution to each of the tubes. (If fluorometer is of a type that requires tubular cuvettes, then carry out all the reactions in matched sets of these cuvettes.) To one of these tubes, add one millilitre of the standard riboflavin solution and mix, and to another, add one millilitre of water and mix. Add one millilitre of acetic acid to both the tubes and mix. Add, with mixing, 0·5 ml of potassium permanganate solution (quantity may be increased for sample solutions that contain excess of oxidizable material but not more than 0·5 ml in excess of that required for complete oxidation of foreign matter should be added). Allow to stand for two minutes. To each of the tubes, add with mixing 0·5 ml of the hydrogen peroxide solution, whereupon the permanganate colour gets destroyed within ten seconds. Shake the tubes vigorously until excess of oxygen is expelled. If gas bubbles remain on the sides of the tubes after the foaming ceases,

remove by tipping the tubes so that the solution flows slowly from end to end.

NOTE — In case of frothing, a drop of alcohol, acetone or *n*-octanol may be added.

4.3.2.2 In the fluorometer, measure fluorescence of the sample solution containing the added one millilitre of the standard riboflavin solution and call this reading as *A*. Next measure fluorescence of the sample solution containing one millilitre of added water, and call this reading as *B*. Add to the same tube with added water, with mixing, 20 mg of powdered sodium hydrosulphite (*see* Note). Measure fluorescence within five seconds, and call this reading as *C*.

NOTE — The sodium hydrosulphite shall be of high purity and kept from undue exposure to light or air. A quantity appreciably in excess of 20 mg may reduce foreign pigments and fluorescing substances or both, thereby causing erroneous results. Suitability of the sodium hydrosulphite may be checked as follows:

To each of two or more tubes, add 10 ml of water and 1 ml of the standard riboflavin solution containing 20 μg of riboflavin per millilitre and proceed as in **4.3.2.1** with respect to the addition of acetic acid, potassium permanganate solution and hydrogen peroxide solution. Then, upon addition with mixing of 8 mg of sodium hydrosulphite, the riboflavin should be completely reduced in not more than five seconds.

4.4 Calculation

4.4.1 Calculate the riboflavin content of the samples on the basis of aliquots taken as follows:

$$\text{mg of riboflavin/ml of the final sample solution} = \frac{B-C}{A-B} \times \frac{1}{10} \times \frac{1}{1000}$$

(value of $\frac{B-C}{A-B}$ shall not be less than 0·66 and not more than 1·5).

4.4.2 Express the results as riboflavin mg/100 g (on dry basis or wet basis, as the case may be).

5. MICROBIOLOGICAL METHOD

5.0 Principle — The micro organism *Lactobacillus casei*, NCTC* No. 6375 (ATCC† No. 7469) has a specific requirement for riboflavin for its growth and lactic acid production. The growth response on a defined medium complete in all respects, except the vitamin under test, is proportional to the concentration of the vitamin added to the medium, up to a certain range.

*National Collection of Type Cultures.
†American Type Culture Collection.

5.1 Apparatus

5.1.1 *Incubator* — maintaining uniform temperature in the range 30° to 37°C (±0·5°C). A water-bath will also serve the purpose.

5.1.2 *Autoclave* — large enough to admit culture tubes in their racks and capable of accurate adjustment of pressure.

5.1.3 *Bacteriological Tubes (Rimless)* — of size 15×150 mm or 25×200 mm.

5.1.4 *Cotton Plugs or Aluminium Caps* — to fit these tubes.

5.1.5 *Culture Tube Racks* — to hold vertically a total of 120 tubes and so designed as to permit free circulation of air. Metal racks resistant to rusting are required.

5.1.6 *Inoculating Needle and Loop* — made of platinum or nichrome steel.

5.1.7 *Hypodermic Syringe* — 5 to 10 ml for inoculating the tubes.

5.1.8 *Refrigerator* — of sufficient capacity to hold reagents, media, stock solutions and the culture.

5.1.9 *Centrifuge* — laboratory model, to hold test-tubes, electrically operated.

5.1.10 *Sterilizing Can* — for pipettes.

5.1.11 *Burette* — 25/50 ml, graduated to 0·1 ml.

5.1.12 *p*H *Meter or Lovibond Comparator* — with disc for adjustment of *p*H between 6 and 8.

5.1.13 *Conical Flasks* — 125- or 100-ml.

5.1.14 *Volumetric Flasks* — 100-ml.

5.1.15 *Volumetric Flask* — 2-litre.

5.1.16 *Graduated Pipettes* — 5- or 10-ml.

5.1.17 *Volumetric Pipettes* — 5-, 10- and 20-ml.

5.1.18 *Other Apparatus* — like funnels, flasks, glass-stoppered cylinders and bottles as necessary. Automatic dispenser titrator, if available, will be useful.

5.2 Reagents

5.2.0 *General* — The principal reagent is a basal medium prepared by mixing a number of stock solutions and chemicals in definite proportions. The stock solutions are to be preserved in the cold (refrigerator) in the dark with 0·1 percent chloroform and 0·5 percent toluene to prevent microbial growth. The solutions should be protected from light, particularly the vitamin solutions, and extract of food materials to be assayed.

5.2.1 *Salt Solution A* — Dissolve 25 g of dipotassium phosphate (K_2HPO_4) and 25 g of monopotassium phosphate (KH_2PO_4) and dilute to 500 ml with water. Store under toluene.

5.2.2 *Salt Solution B* — Dissolve the following and dilute to 500 ml with water:

Sodium chloride (NaCl)	0·5 g
Ferrous sulphate ($FeSO_4$, $7H_2O$)	0·5 g
Manganese sulphate ($MnSO_4$, $4H_2O$)	0·5 g
Magnesium sulphate ($MgSO_4$, $7H_2O$)	10·0 g

Add 5 drops of concentrated hydrochloric acid and store under toluene.

5.2.3 *Hydrochloric Acid* — 0·1 N (approx). Dilute 8·5 ml of concentrated hydrochloric acid to 1 litre with water.

5.2.4 *Stock Riboflavin Solution A* — 25 µg riboflavin per millilitre in 0·02 N acetic acid. The solution may be prepared as follows:

Weigh accurately 50 mg reference standard riboflavin and transfer quantitatively to a 2-litre volumetric flask, preferably amber coloured. Add about 1 500 ml of water, 2·4 ml of glacial acetic acid and warm in a water-bath to aid solution. Cool to room temperature and make up to volume. Preserve under toluene in the cold.

5.2.5 *Stock Riboflavin Solution B* — 10 µg/ml in 0·002 N acetic acid. Dilute 40 ml of stock riboflavin solution A, to 100 ml with water. Preserve as before.

5.2.6 *Riboflavin Working Standard* — 0·1 µg/ml. Dilute 1 ml of stock solution B to 100 ml with water. Prepare this immediately before use.

5.2.7 *Photolyzed Peptone* — Dissolve 40 g of Difco/Bacto or equivalent peptone in 250 ml of water and 20 g of sodium hydroxide in 250 ml of water. Mix these two solutions. Allow to stand for 24 hours in the room. Expose the solution to the light of a 100-watt bulb kept at a distance of about 45 cm for 18 hours, to ensure destruction of all riboflavin in the peptone. At the end of the exposure, neutralize the solution with glacial acetic acid (25 to 28 ml) and add 14 g of anhydrous sodium acetate. Make up to 800 ml with water and preserve under a layer of sulphur-free toluene. This solution generally keeps for 14 days in a refrigerator, but if precipitate occurs or if the solution becomes cloudy before this time, it should be discarded.

5.2.8 *Cystine Solution* — 0·1 percent (*w*/*v*). Suspend 1 g of *l*-cystine in 20 ml of water. Add concentrated hydrochloric acid dropwise (about 10 ml) until the crystals dissolve. Add water to make up to a litre. Preserve under toluene.

5.2.9 *Yeast Supplement* (*Riboflavin-Free*) — Dissolve 100 g of Difco/Bacto or equivalent yeast extract or autolyzed yeast in 500 ml of water.

Add 150 g of basic lead acetate dissolved in 500 ml of water to this. Mix the two solutions and adjust the *p*H to approximately 10, with concentrated ammonium hydroxide using an external indicator. Filter through a Buchner funnel. Adjust the *p*H to about 6·5 with glacial acetic acid and pass hydrogen sulphide to remove the lead, as lead sulphide. Filter the black precipitate, adjust the volume of the filtrate to 1 000 ml. Excess hydrogen sulphide may be removed by passing air for sometime or removing 200 ml of water under vacuum. Preserve with toluene and chloroform in the refrigerator.

5.2.10 *Basal Medium Stock Solution (for 100 Tubes)* — Mix the following:

Photolyzed peptone	100 ml
0·1 percent cystine	100 ml
Yeast supplement solution	20 ml
Salt solution A	10 ml
Salt solution B	10 ml
Glucose, anhydrous	10 g

Dissolve the glucose in the mixture of solutions, adjust *p*H to 6·8 with 1 N sodium hydroxide and make up to 500 ml with water.

5.2.11 *Enriched Agar Medium for Stock Culture* — Take 2 g of anhydrous glucose, 1 g of peptone (Difco/Bacto or equivalent), 100 mg of cystine, 1 ml of salt solution A, 1 ml of salt solution B and 3·5 g of Bacto/Difco or equivalent agar. First dissolve all ingredients except agar in about 150 ml of water and adjust *p*H to 6·8 and make up to 200 ml. Add now agar and steam the mixture, till the agar gets dissolved. Dispense into 20 test-tubes, stopper them with cotton plugs and sterilize at 120° to 123°C for 15 minutes.

5.2.12 *Culture Medium for Growing Inoculum* — Dissolve 5 g of peptone, 1 g of yeast extract, 10 g of anhydrous glucose, 10 g of anhydrous sodium acetate, 5 ml each of salt solutions A and B, in 200 ml of water in a beaker. Adjust *p*H to 6·8 with 1 N sodium hydroxide and dilute to 500 ml. Filter and distribute 10-ml quantities to test-tubes, plug with cotton and sterilize at 120° to 123°C for 15 minutes.

5.2.13 *Isotonic Salt Solution* — Dissolve 0·9 g of sodium chloride in 100 ml of water. Distribute 10 ml each into test-tubes, plug with cotton and sterilize as before.

5.2.14 *Sodium Hydroxide* — 1 N. Take 40 g of NaOH pellets and dissolve in one litre of water. Standardize against potassium hydrogen phthalate of known strength.

5.2.15 *Sodium Hydroxide* — 0·1 N. Prepare by diluting 1 N sodium hydroxide 10 times with water.

5.2.16 *Bromothymol Blue Indicator Solution* — Weigh 1 g of bromothymol blue indicator into a small beaker. Add 1·6 ml of 0·1 N

sodium hydroxide and stir the powder with a glass rod, till dissolved. Dilute with water to 250 ml. The solution may also be made by dissolving in a few millilitres of 95 percent ethanol, adding 1·6 ml of 0·1 N sodium hydroxide and then diluting to 250 ml with water.

5.3 Procedure

5.3.0 Use active stab culture of the stock *L. casei* not older than 10 to 14 days for the preparation of inoculum.

5.3.1 *Preparation of Stock Culture*

a) Prepare stab cultures in 2 or more agar stock culture tubes (**5.2.11**) using a pure culture which may be obtained from the National Collection of Type Cultures or from American Type Culture Collection.

b) Incubate for 16 to 24 hours at 37·0° ± 0·5°C.

c) Store in the refrigerator under aseptic condition for not longer than one week before transferring to new stab.

5.3.2 *Preparation of Inoculum*

a) Transfer aseptically the culture cells from stock culture to a sterile tube of inoculum culture medium (**5.2.12**).

b) Incubate this culture for 12 to 18 hours at 37°C. Inoculum older than 24 hours should not be used for assay. In case of emergency, the inoculum may be refrigerated for 24 hours.

c) Secure the cotton plug with rubber band or adhesive tape and centrifuge for a few minutes (15 minutes).

d) Decant the supernatant liquid and suspend the cells aseptically in 20 ml of sterile isotonic salt solution.

e) The centrifuging may be repeated and the cells again resuspended in sterile salt solution if desired.

f) Fill a sterile syringe with resuspended cells and use at once. Twenty millilitres of inoculum will be sufficient for 200 assay tubes. Alternatively, a sterile pipette or a sterile glass tube drawn out to a capillary may also be used in place of a syringe.

5.3.3 *Preparation of Sample-Extraction and Hydrolysis*

a) Into a 100/125-ml conical flask, weigh a homogeneous sample containing 10 µg or more of riboflavin, add 50 ml of 0·1 N hydrochloric acid and autoclave for 15 minutes at 120° to 123°C.

b) Cool to room temperature, adjust *p*H to 4·5, transfer to a 100-ml volumetric flask, make to volume and filter.

c) Measure 50 ml (or aliquot containing about 5 μg of riboflavin) of the filtrate into a 100-ml volumetric flask, adjust *p*H to 6·8 and dilute to 100 ml.

d) Use five levels of sample extract ranging from 0·05 to 0·25 μg; each tube varying by not less than 0·5 ml between two serial additions.

5.3.4 *Preparation of Standard Tubes*

a) Add, in duplicate tubes numbered serially, aliquots of the working standard riboflavin solution.

b) Add sufficient water to bring the volume in each tube to 5·0 ml.

c) Into each tube, add 5 ml of basal medium stock solution and mix well by rotating in the palm of the hand.

d) Add 5 ml of water and 5 ml of basal medium stock solution into two tubes to serve as blank.

e) For establishing standard curve, 0·05 μg, 0·10 μg, 0·15 μg, 0·2 μg and 0·25 μg of riboflavin are recommended. Automatic pipettes may be used.

f) Cover each tube with cotton plug or aluminium cap and sterilize at 115°C for 10 minutes.

g) Cool the tubes in the dark before inoculation.

5.3.5 *Preparation of Assay Tubes*

a) To duplicate tubes, numbered or marked serially, add at five levels of aliquot (0·5 to 5·0 ml) of the test extract of food material (approximately within 0·05 to 0·20 μg).

b) Add sufficient water to bring the volume to 5 ml.

c) To each of the tubes, add 5·0 ml of the basal medium stock solution and mix each tube by rotating in the palm.

d) Plug with cotton or cover with aluminium caps.

e) Sterilize at 115°C for 10 minutes.

5.3.6 *Inoculation and Incubation*

a) Cool all tubes to the incubation temperature. Small difference in temperature of the various tubes at the start of the assay influences the rate of growth.

b) Aseptically inoculate each tube with one drop of the inoculum. It is very convenient to do this in a chamber or hood.

c) Incubate at 37°C for approximately 72 hours. The temperature should be kept uniform throughout.

5.3.7 *Titration*

a) Transfer the contents of each tube into 100/125-ml conical flask. Rinse each tube twice with about 10 ml of water and add the rinsing to the flask.

b) Add about 0·2 ml of the 0·1 percent bromothymol blue (2 to 3 drops) and titrate with 0·1 N sodium hydroxide to a green colour, *p*H about 6·8.

c) Hold one flask for reference colour for 10 titrations and then substitute with another new flask to get the correct point.

d) Titrate the blank also to the same end point.

5.3.8 *Calculation*

a) Draw a standard curve for the assay by plotting the millilitres of 0·1 N sodium hydroxide against concentration of standard vitamin added per tube, after subtracting the value for blank.

b) Determine the vitamin content of the tubes in the unknown series by interpolation of their titre values on the standard curve.

c) Calculate the vitamin content of test material from the average of the values for 1 ml of test solution obtained from not less than three sets of the tubes which do not vary by more than 10 percent from the average, using the formula:

$$\text{Micrograms of riboflavin/g food sample (on dry basis or wet basis as the case may be)} = \frac{\text{Average } \mu g/ml \times \text{Volume} \times \text{Dilution factor}}{\text{Weight of sample in grams}}$$

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Telegrams Manaksanstha

Branch Offices

Telegrams Manaksanstha

Syndicate Bank Buildings, Gandhinagar	Bangalore 9	Telephone	—
534 Sardar Vallabhbhai Patel Road	Bombay 7	,,	35 69 44
5 Chowringhee Approach	Calcutta 13	,,	23-08 02
5-9-201/2 Chirag Ali Lane	Hyderabad 1	,,	3 23 35
117/418 B Sarvodaya Nagar	Kanpur 5	,,	82 72
54 General Patters Road	Madras 2	,,	8 72 78

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